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# The Effects of Testosterone on Calling Behavior in Green Treefrogs, *Hyla cinerea*

Brandon Bunol

*University of Mississippi. Sally McDonnell Barksdale Honors College*

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THE EFFECTS OF TESTOSTERONE ON CALLING BEHAVIOR IN GREEN  
TREEFROGS, *HYLA CINEREA*

by  
Brandon Bunol

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of  
the requirements of the Sally McDonnell Barksdale Honors College.

Oxford 2015

Approved by

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## ABSTRACT

BRANDON JOSEPH BUNOL: The Effects of Testosterone on Calling Behavior in  
Green Treefrogs, *Hyla cinerea*  
(Under the direction of Dr. Christopher Leary)

Circulating androgen levels can have a profound effect on the expression of elaborate male traits and courtship behaviors that are important in attracting females. For example, elevated androgens are necessary for the expression of vocal displays used by male anurans (frogs and toads) to attract mates. However, the nature of the relationship between androgen levels and the extent, or magnitude, of vocalization in male anurans is unclear. For example, androgens may have a threshold effect on vocalization wherein elevation in androgens is required for vocal expression, but elevations above threshold do not mediate an increase in the magnitude or extent of vocalization. Alternatively, androgens may have graded effects on vocalization, wherein elevations above threshold levels required for vocal expression correspond to an increase in the extent or magnitude of vocalization. Numerous androgen-based models of sexual selection propose that as testosterone levels increase, the extent and/or magnitude of elaborate male traits increases in a graded, dose-dependent (linear) manner. This relationship provides the basis for ‘honest signals’ (e.g., signals that confer ‘good genes’). I examined how circulating androgen levels relate to vocal parameters known to be important in mate selection in male green treefrogs, *Hyla cinerea*. Results provided no evidence of a graded effect of testosterone on vocalization. Hence, circulating androgens do not appear to mediate

changes in vocalization important in mate selection in a graded manner, contrary to the predictions of theoretical models.

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## LIST OF ABBREVIATIONS

T	Testosterone
DHT	Dihydrotestosterone
ICHH	Immunocompetence Handicap Hypothesis
EHV	Energetic-Hormone Vocalization Model
CORT	Corticosterone

## INTRODUCTION

Circulating hormone levels can have a profound influence on elaborate male traits and behaviors associated with mate acquisition. In particular, androgens (e.g., testosterone, T and dihydrotestosterone, DHT) are known to mediate the expression of male secondary sex characteristics and reproductive behavior (*reviewed* by Adkins-Regan, 2005). For example, numerous studies have shown that the expression of elaborate male traits (i.e., comb development and plumage color in some birds) and male courtship behaviors (i.e., vocalization in songbirds and amphibians) are androgen dependent (McGlothlin et al., 2008; Fusani, 2008; Wilczynski et al., 2005; Moore et al., 2005). Although androgens are known to mediate the expression of many elaborate male traits and male sexual behavior, the nature of this relationship is less clear (Hews and Moore, 1997; Leary & Knapp, 2014). For example, androgens may mediate the expression of traits in a graded, dose-dependent (linear) manner, in which elevations above the minimum threshold required for expression of the trait causes a further increase in the magnitude or extent of trait expression (Fig. 1A; Hews and Moore, 1997). In contrast, androgens may mediate the expression of elaborate traits in a threshold (non-linear) manner. Under a threshold scenario, a trait is fully expressed when androgen levels exceed a minimum threshold, but elevations above threshold do not further increase the expression of the trait (Fig. 1B; Hews and Moore, 1997).

Graded versus threshold effects of hormones have become critical in understanding the evolution of the endocrine system, elaborate male traits and male sexual behavior. For example, several prominent and highly influential endocrine-based models of sexual selection propose that female preferences for more elaborate male traits select for increased androgen levels in males (*reviewed* by Leary and Knapp, 2014). Under a dose-dependent (graded) scenario, female preferences for more elaborate male traits are expected to drive concordant directional selection on circulating androgen levels. However, under a threshold scenario, female preferences for more elaborate male traits may not result in concordant directional selection on circulating androgen levels via mate choice by females. To assess the nature of the relationship between circulating androgen levels and elaborate male traits, I examined how circulating levels of androgens (i.e., testosterone) affect vocal parameters that are known to be important in mate selection in green treefrogs, *Hyla cinerea*.

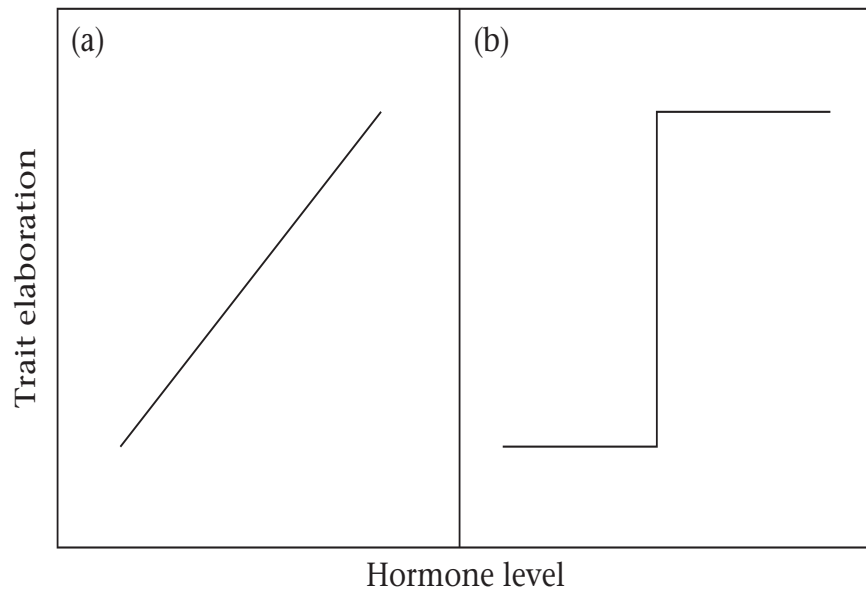


Figure 1: Graded (a) versus threshold effects (b) of hormones on trait elaboration  
(modified from Hews and Moore, 1997)

## **Androgen-Based Models of Sexual Selection and Trait Elaboration**

The Immunocompetence Handicap Hypothesis (ICHH; Folstad and Karter, 1992) has been pivotal in directing research focusing on androgenic effects on elaborate male traits and courtship behavior. The ICHH predicts that androgens have a graded, dose-dependent effect on the magnitude of trait elaboration and/or courtship behavior that increase a male's overall attractiveness (Folstad and Karter, 1992). However, the ICHH predicts that there will be negative consequences associated with elevations in testosterone levels, such as immunosuppression, which decreases a male's survivorship (Folstad and Karter, 1992). Therefore, only males that possess 'good genes' (i.e., genes that confer a male's resistance to pathogens), can afford the cost of high androgen levels. Hence, circulating androgen levels provide the basis for 'honest signals' used by females to select mates. Such 'honest signals' would presumably provide females with an accurate assessment of indirect benefits that females may gain by mating with males with more elaborate male traits (i.e., males with high androgen levels).

One major criticism of the ICHH model is the assumption that androgen levels are tightly coupled to the magnitude of trait expression i.e., androgens have a graded, dose-dependent effect on the magnitude or extent of elaborate male traits (*reviewed* by Hews and Moore, 1997; Leary & Knapp, 2014). If the magnitude of the trait does not accurately track variation in circulating androgen levels (i.e., as with a threshold scenario), then males could down regulate androgen levels to threshold levels required to fully express the trait and avoid the negative consequences associated with elevated androgen levels without negatively affecting the expression of the trait (Hews and Moore, 1997; Leary and Knapp, 2014). Under a threshold scenario, circulating androgen levels

are not a reliable predictor of signal quality and are thus unlikely to maintain the honesty of the signal (*reviewed* in Hews and Moore, 1997; Leary and Knapp, 2014).

Variation in vocal quality in anuran amphibians (frogs and toads) is also thought to be related to graded effects of androgens. The Energetics-Hormone Vocalization (EHV) model, for example, proposes that androgens have a dose-dependent effect on vocal effort (i.e. the total amount of energy invested in vocalization) which is characteristically under strong directional selection via mate choice by females (Emerson, 2001). Thus, as circulating androgens levels increase, a male is predicted to invest more energy in vocalization. However, to meet the rising energetic demands associated with increased vocal effort, a simultaneous increase in glucocorticoids (i.e. corticosterone, CORT, the primary glucocorticoid in amphibians) occurs until a maximum level is reached that inhibits the production of androgens, which causes males to cease vocalization entirely (Fig. 2; Emerson, 2001). The EHV model thus predicts that variation in vocal quality is mediated by circulating androgen levels and that increasing levels of testosterone should mediate an increase in vocal effort. In contrast, under a threshold scenario, some minimum level of androgens are required to mediate the expression of calling behavior, but any increase in circulating androgens above this minimum threshold would not result in an increase in vocal effort. I used the male green treefrog, *Hyla cinerea*, as a model system to test predictions of the ICHH and the EHV model (Folstad and Karter 1992; Emerson, 2001).

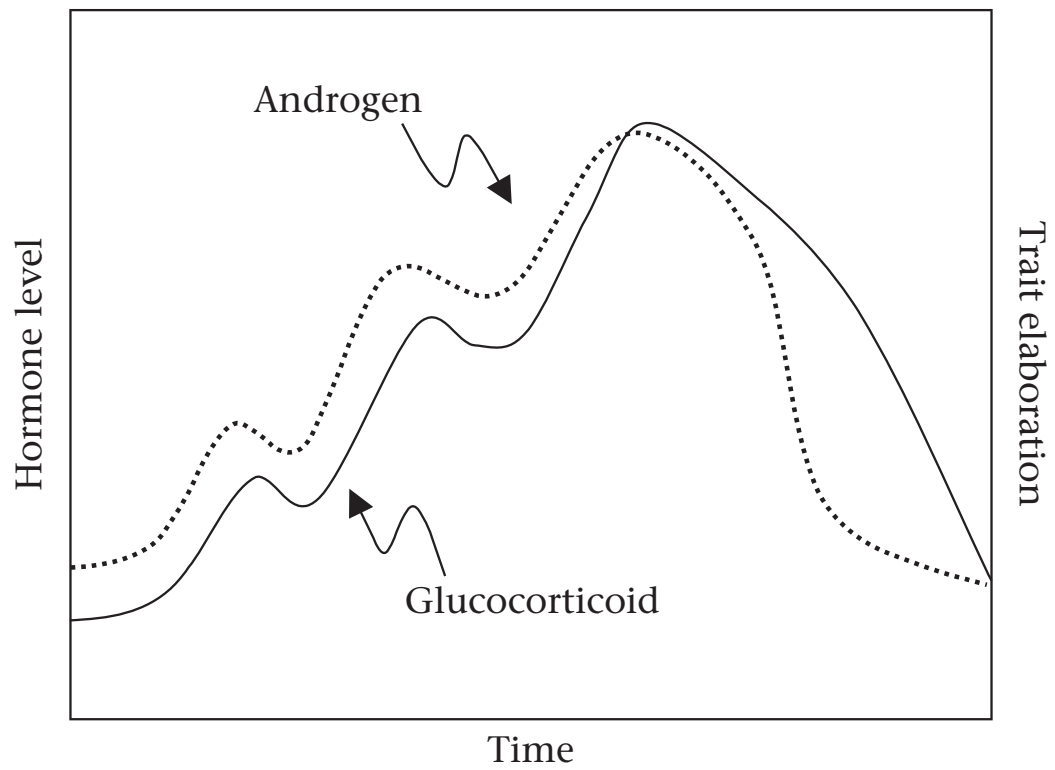


Figure 2: The Energetics-Hormone Vocalization model (modified from Emerson, 2001).

## **Female Preferences in Anurans**

Preferences of female anurans are often based on different static and dynamic properties of male advertisement calls (Fig. 3; Gerhardt, 1991). Static properties are relatively invariable within and among individuals and are often subject to stabilizing selection via mate choice by females. For example, Gerhardt (1991) demonstrated that female *H. cinerea* prefer a call frequency that reflects the mean of the population and stabilizes selection on this call parameter. In contrast, dynamic properties are highly variable within and among individuals and are under strong directional selection via mate choice by females (Gerhardt, 1991). For example, Gerhardt (1991) found that female *H. cinerea* preferred synthetic calls broadcast at a faster call rate. Call duration is also generally highly variable and under strong directional selection in anurans; however, in *H. cinerea* it is less variable and appears to be under weak directional selection via mate choice by females. For instance, female *H. cinerea* prefer calls that exceed the longest duration of natural populations, but do not prefer calls that are extremely long (Gerhardt, 1991). The EHV model predicts that elevations in circulating androgen levels should result in a concordant increase in call duration and/or call rate that contribute to an increase in vocal effort; therefore, selection of these parameters via female preferences should drive directional selection on androgen levels.



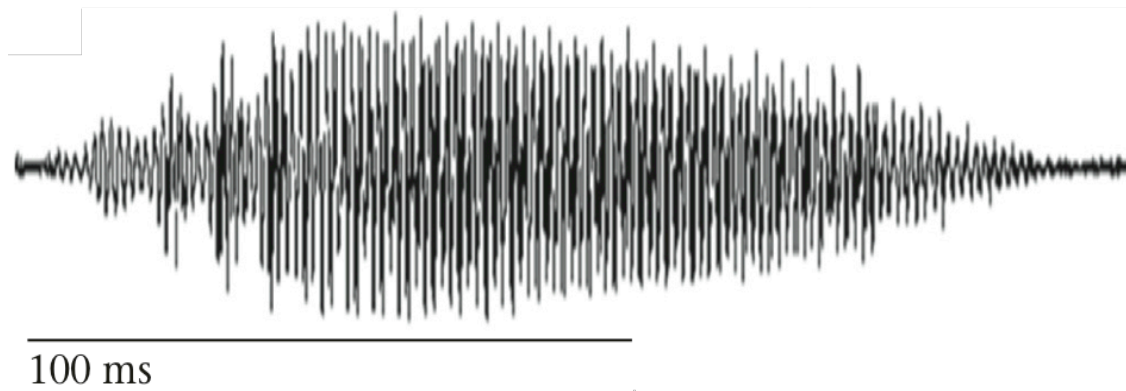


Figure 3: Waveform representation of an advertisement call of male *Hyla cinerea*.

## **Androgens Examined in Current Study**

Amphibians produce two main circulating androgens that include testosterone, T and dihydrotestosterone, DHT. T can be aromatized to estradiol and act on estrogen receptors; T can also interact with androgen receptors directly or through conversion to DHT by cytoplasmic enzymes (Swerdloff and Wang, 1998). In contrast, DHT cannot be converted into estradiol or T. I focused on the effects of T on vocalization in *H. cinerea* because sex steroid-mediated effects of androgens in males often occur through the conversion of T to estradiol at target tissues (Peterson et al., 2013). Based on the underlying predictions of the EHV model and the ICHH, T administration is expected to increase the attractiveness of males by mediating an increase in vocal effort (Emerson, 2001).

## METHODS

### Study Site

All research was conducted at the University of Mississippi (UM) Field Station. Observations were conducted during the 2014 breeding season (May to July). Chorusing behavior was observed nightly between the hours of 2100 and 2300 at multiple ponds.

### Field Data Collection

Vocal observations were made on vocalizing *H. cinerea* in natural choruses using low-powered LED headlamps. To assess how androgens potentially influence vocalization, I first acquired individual vocal recordings of 30-50 consecutive calls using a Marantz PMD recorder equipped with a Sennheiser microphone. Individual males were then captured by hand and a blood sample was rapidly acquired in less than 2 minutes via cardiac puncture. Blood samples were then stored on ice until they were returned to the laboratory (<6 hours). These samples were centrifuged for 12 minutes at 3000 revolutions/min to separate the plasma from the blood. The plasma was then stored at -20°C until assayed for steroid hormones.

After capture, individuals were measured from tip of the snout to the distal end of the ischium (to the nearest 1mm) and weighed (to the nearest 0.1g) on a portable OHAUS digital scale. Once males were returned to the laboratory, they were individually tattooed on the ventral side using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL,

U.S.A.). These tattoos were used to identify individuals throughout the breeding season to avoid sampling the same individuals throughout the course of the study.

Captured males were then injected in the hind limb musculature with either 100µl castor oil (controls; n = 9) or 16µg of T in 100µl castor oil vesicle (n = 10). Males were then kept in 10-gallon aquaria with water and fed crickets ad libitum. Captured individuals were released at the site of capture 24-48 hours later.

Following release, vocal observations were made on treated males. If males called subsequent to release, a series of approximately 30-50 calls were recorded and the individual was captured and bled a second time via cardiac puncture (< 2 min).

### **Vocal Analysis**

Recorded calls were analyzed using Raven Pro 1.4 (The Cornell Bioacoustics Laboratory). Call duration, dominant carrier frequencies, and intercall duration, were measured from 10 consecutive calls prior to and subsequent to treatment. Once these call parameters were measured, vocal effort was calculated using the following equation:

$$Vocal\ Effort = \frac{Call\ duration\ (ms)}{Call\ duration\ (ms) + intercall\ duration\ (ms)}$$

### **Hormone Assays**

Levels of circulating hormones were measured using column chromatography followed by radioimmunoassay. Hormone separation and quantification of hormone concentrations followed the protocol described by Leary and Harris (2013). Briefly, plasma samples were incubated overnight with radiolabeled hormone (PerkinElmer, Inc. Hebron, Kentucky) for determination of recoveries for each sample. Steroids were then

extracted from plasma using diethyl ether, dried under nitrogen gas at 40 °C, and resuspended in 10% ethyl acetate in isooctane. Samples were then loaded onto diatomaceous earth columns containing a 3:1 diatomaceous earth: distilled water “glycol trap” and a 1:1 propanediol: ethylene glycol mixture. Mixtures of 10%, 20%, and 52% ethyl acetate in isooctane were then used to collect DHT, T and CORT, respectively. Fractions were dried under nitrogen and resuspended in phosphate buffered saline containing 0.3% gelatin for use in radioimmunoassay. Testosterone antibody was obtained from Fitzgerald Industries International, Inc. (Acton, Massachusetts) and used for both T and DHT assays. Corticosterone antibody was purchased from MP Biomedicals, LLC (Solon, Ohio). All samples were assayed in duplicate.

Plasma samples were analyzed for DHT, T and CORT levels in 2 assays. Blood samples that were taken from the same individual were run in the same assay.

### **Statistical Analysis**

Analyses of call parameters and hormone concentrations were conducted using Sigma Plot 11.0 (Systat Software Inc., Chicago, IL, U.S.). Linear regression analysis was used to assess the relationship between circulating hormone levels and call attributes (i.e., dominant carrier frequency, call duration, intercall duration and vocal effort) prior to treatment for T injected individuals (n = 10) and castor oil injected individuals (n = 9). An additional 12 individuals were also used for this analysis; these males were used as part of another study that examined the effects of dihydrotestosterone on calling behavior. Paired t-tests were run to examine the effects of treatment on circulating hormone levels and vocalization.

## RESULTS

### Relationship between Testosterone and Call Parameters before Treatment

Circulating levels of T prior to injections were examined in relation to vocal parameters (i.e., low and high carrier frequencies, call duration, intercall duration and vocal effort) that are important in mate selection by female *H. cinerea*. Circulating T levels were not significantly related to low carrier frequency ( $F_{1,29}=0.51$ ,  $p=0.47$ ,  $r^2=0.017$ ; Fig. 4) or high carrier frequency of the advertisement call ( $F_{1,29}=0.172$ ,  $p=0.68$ ,  $r^2=0.006$ ; Fig. 5). There was also no evidence that circulating T levels were significantly related to call duration ( $F_{1,29}=0.256$ ,  $p=0.61$ ,  $r^2=0.009$ ; Fig. 6), intercall duration ( $F_{1,29}=0.421$ ,  $p=0.51$ ,  $r^2=0.014$ ; Fig. 7), or vocal effort ( $F_{1,29}=0.138$ ,  $p=0.71$ ,  $r^2=0.005$ ; Fig. 8).

### Hormone Levels

Circulating hormone levels (DHT, T and CORT) were examined prior to and subsequent to treatment. T injections resulted in a significant increase in circulating levels of T ( $t_9=-6.959$ ,  $p<0.001$ ; Fig. 9a), indicating that injections were effective at elevating T levels. T levels did not differ prior to and subsequent to castor oil injections ( $t_8=1.255$ ,  $p=0.24$ ; Fig. 9a). Levels of DHT significantly decreased subsequent to castor oil injections ( $t_8=2.832$ ,  $p=0.02$ ; Fig. 9b) and T injections ( $t_9=2.632$ ,  $p=0.02$ ; Fig. 9b). CORT levels did not differ significantly prior to and subsequent to T injections ( $t_9=-1.839$ ,  $p=0.09$ ) or castor oil injections ( $t_8=0.325$ ,  $p=0.24$ ; Fig. 9c).

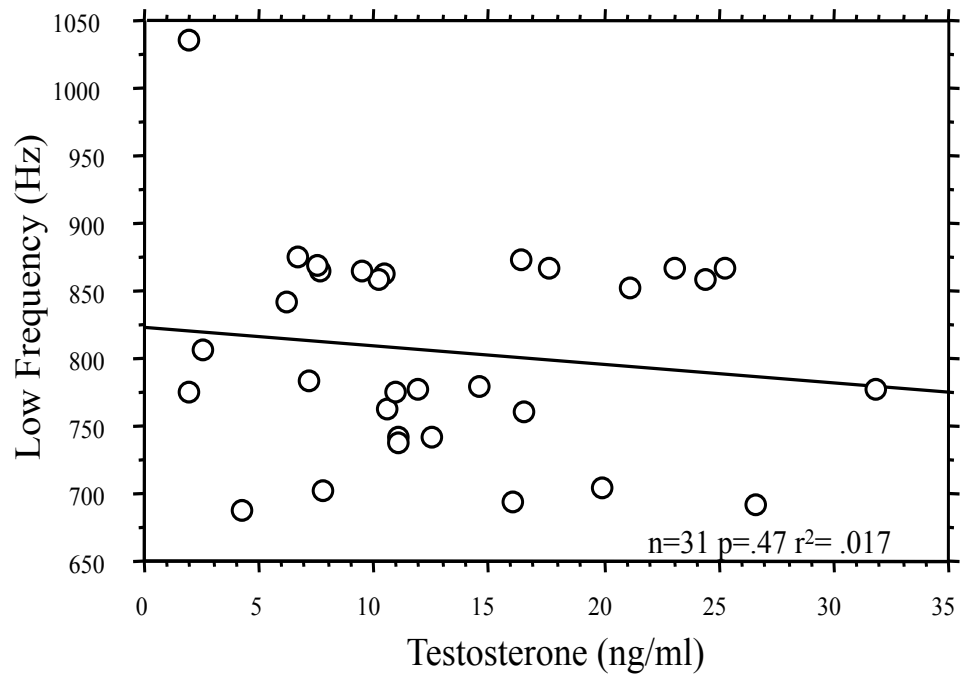


Figure 4: Linear regression showing the relationship between circulating T levels and low carrier frequency of the advertisement call in *Hyla cinerea*.

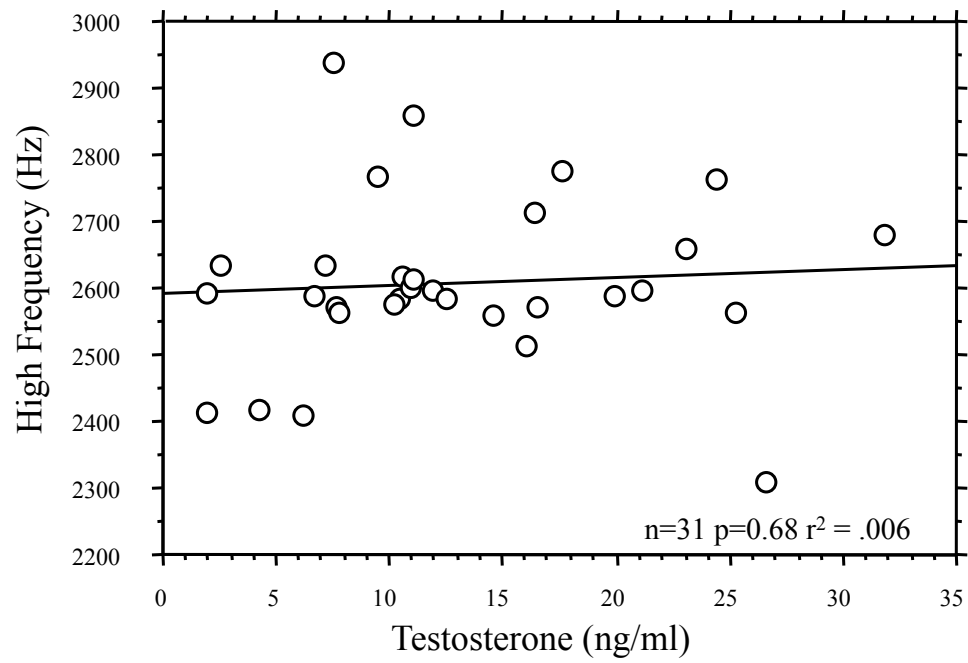


Figure 5: Linear regression showing the relationship between circulating T levels and high carrier frequency of the advertisement call in *Hyla cinerea*.



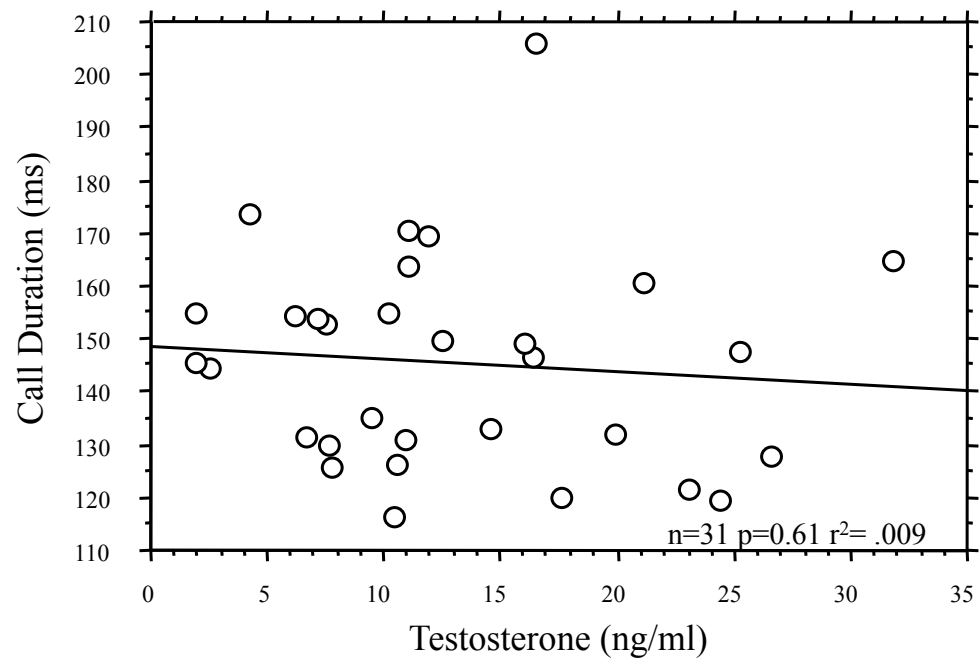


Figure 6: Linear regression showing the relationship between circulating T levels and call duration of the advertisement call in *Hyla cinerea*.

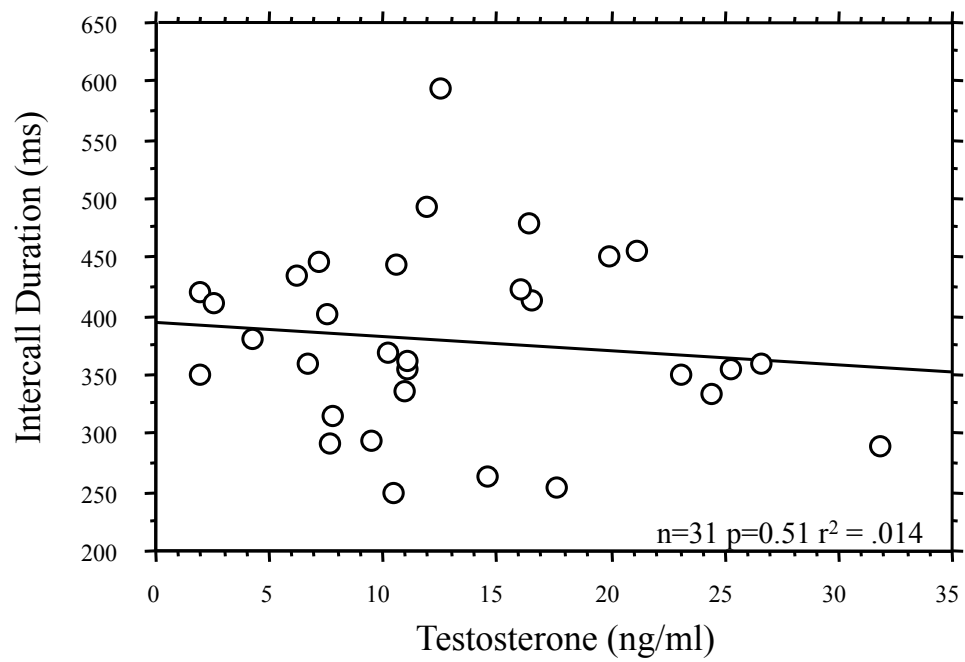


Figure 7: Linear regression showing the relationship between circulating T levels and intercall duration of the advertisement call in *Hyla cinerea*.

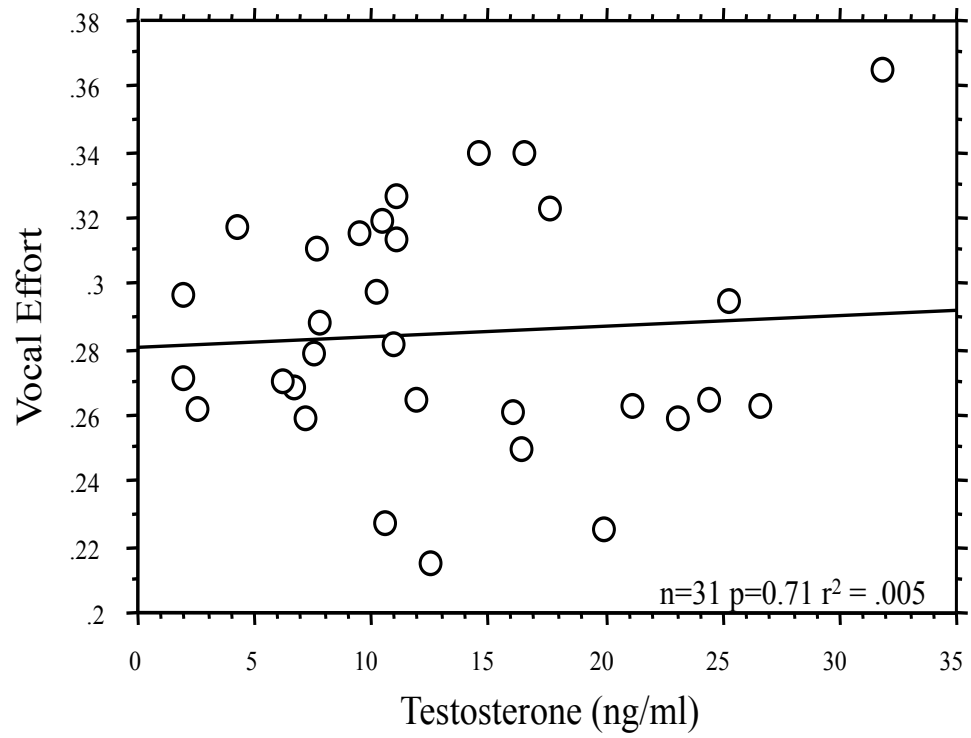


Figure 8: Linear regression showing the relationship between circulating T levels and vocal effort of the advertisement call in *Hyla cinerea*.

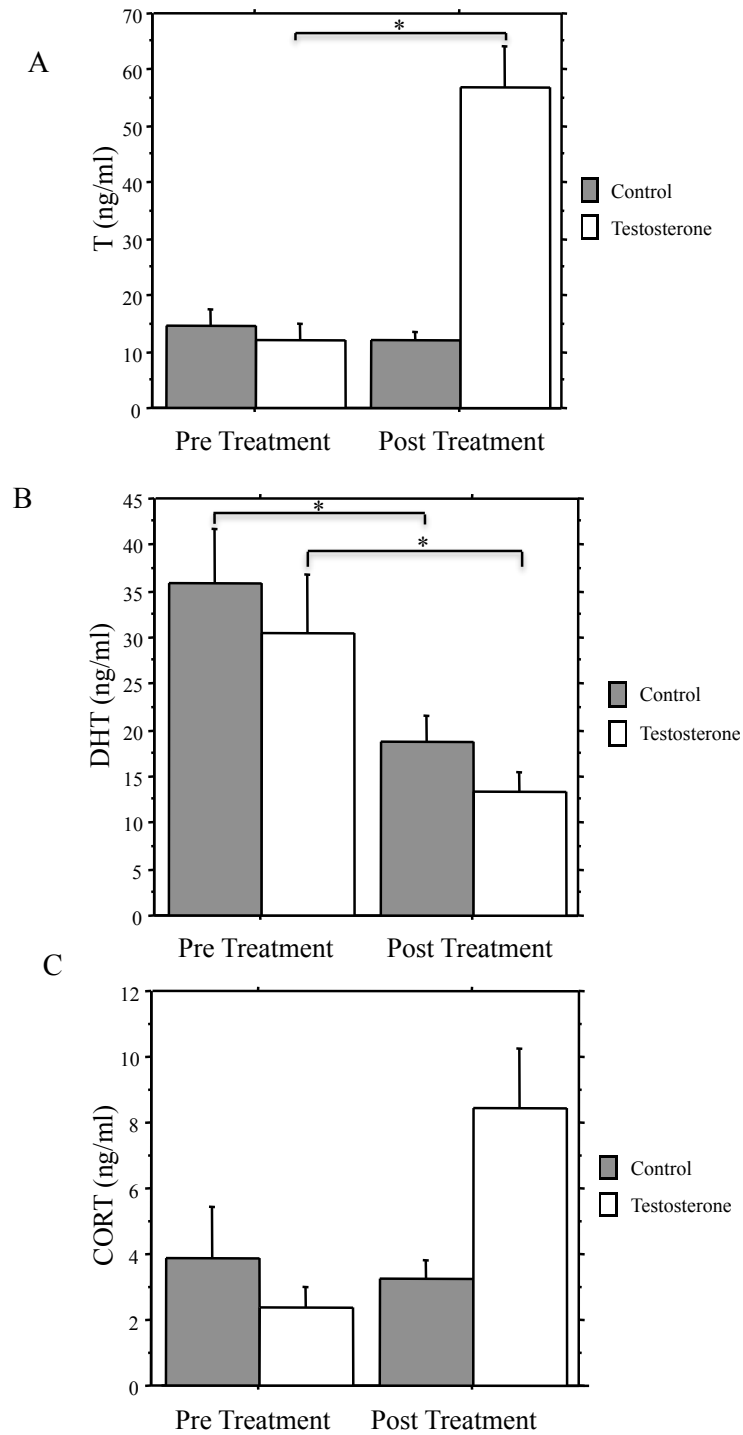


Figure 9: Circulating levels of (A) T, (B) DHT and (C) CORT prior to and subsequent to treatment for the castor oil-injected controls (n = 9) and T-injected group (n = 10).

Whiskers depict standard errors and asterisks denote significant differences.

### Call Parameters before and after Treatment

Call parameters that are important in mate selection by female *Hyla cinerea* were analyzed from vocal recordings prior to and subsequent to treatment to examine the effects of T injections on vocalization. Low carrier frequency increased in males injected with T ( $t_9=-5.910$ ,  $p=0.002$ ), but it did not differ significantly in castor oil-injected controls ( $t_8=0.803$ ,  $p=0.44$ ; Fig. 10). High carrier frequency did not differ significantly in males injected with T ( $t_9=0.809$ ,  $p=0.43$ ; Fig. 11), or males injected with castor oil ( $t_8=0.273$ ,  $p=0.79$ ; Fig. 11). Call duration decreased significantly in T-injected individuals ( $t_9=3.666$ ,  $p=0.005$ ; Fig. 12), but did not differ significantly in castor oil-injected males ( $t_8=1.715$ ,  $p=0.12$ ; Fig. 12). Intercall duration did not differ significantly in males injected with T ( $t_9=-1.855$ ,  $p=0.09$ ) but there was a significant increase in intercall duration in males injected with castor oil ( $t_8=-2.467$ ,  $p=0.03$ ; Fig. 13). A significant decrease in vocal effort was observed in both T-injected males ( $t_9=5.917$ ,  $p=0.002$ ) and castor oil-injected males ( $t_8=3.917$ ,  $p=0.04$ ; Fig. 14).

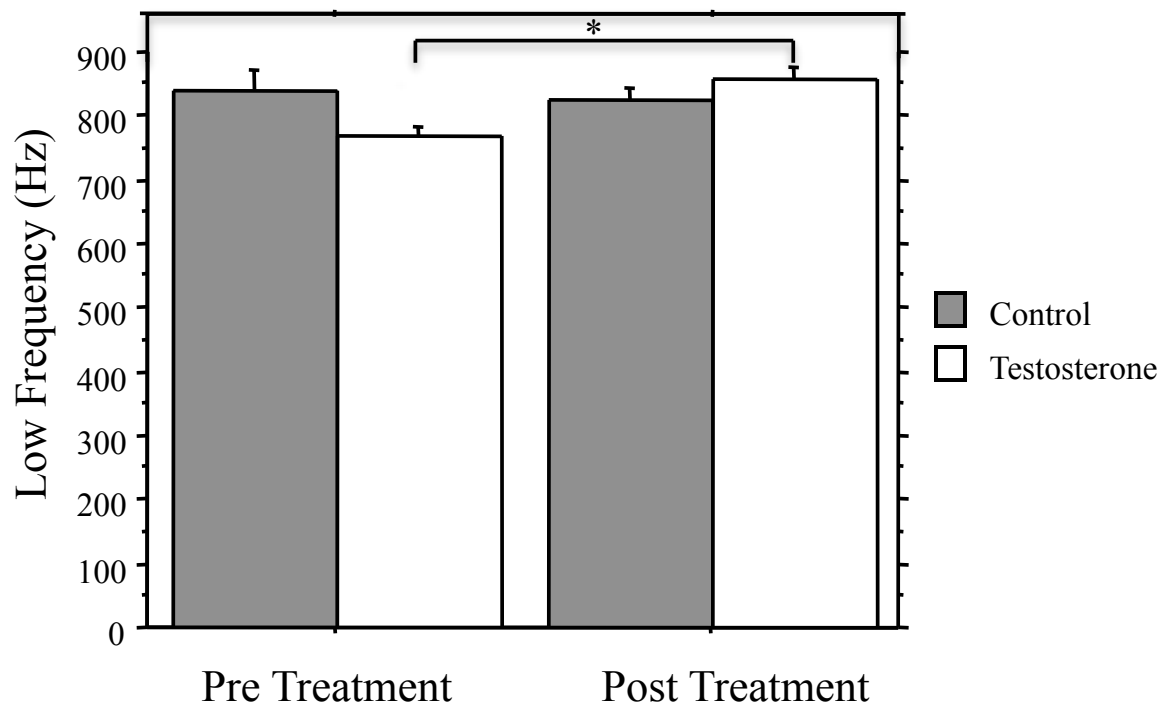


Figure 10: Changes in low frequency (Hz) prior to and subsequent to castor oil (n = 9) or T injection (n = 10) in *H. cinerea*. Whiskers depict standard errors and asterisks denote significant differences.

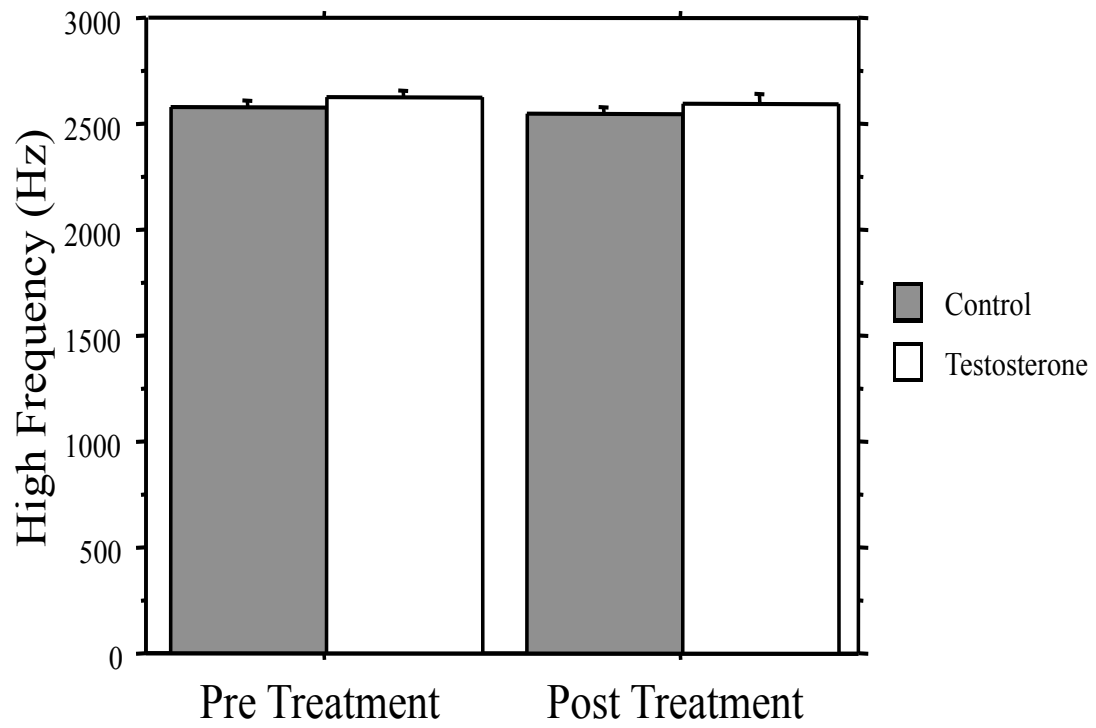


Figure 11: Changes in high frequency (Hz) prior to and subsequent to castor oil (n = 9) or T injection (n = 10) in *H. cinerea*. Whiskers depict standard errors.

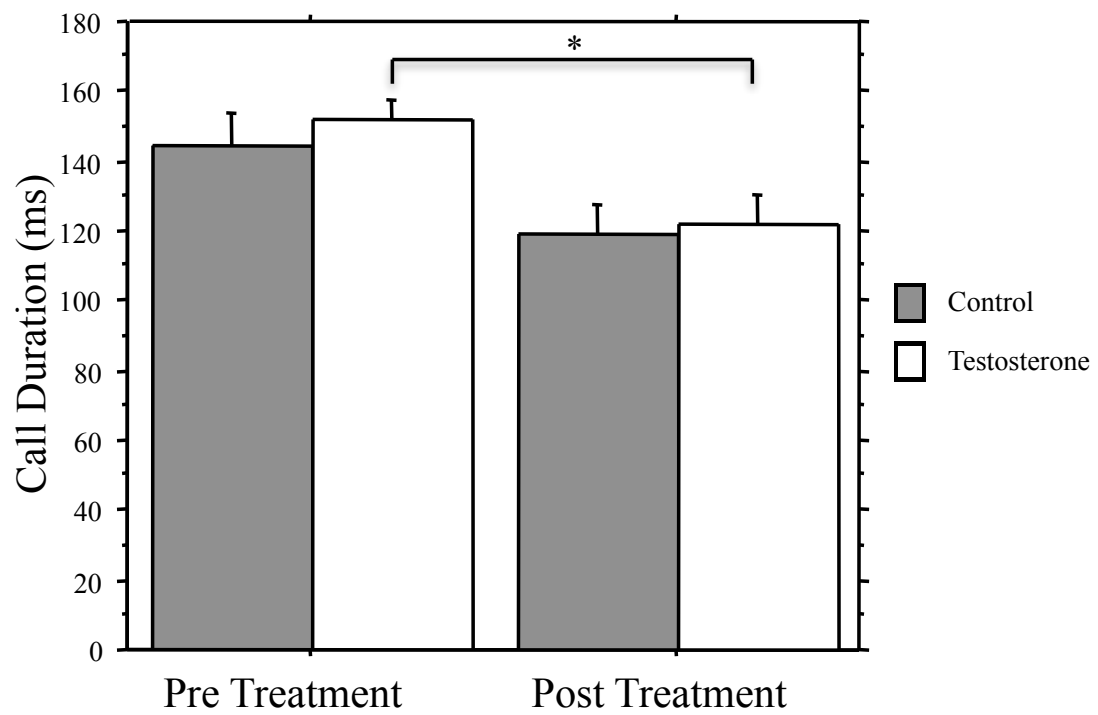


Figure 12: Changes in call duration (ms) prior to and subsequent to castor oil ( $n = 9$ ) or T injection ( $n = 10$ ) in *H. cinerea*. Whiskers depict standard errors and asterisks denote significant differences.



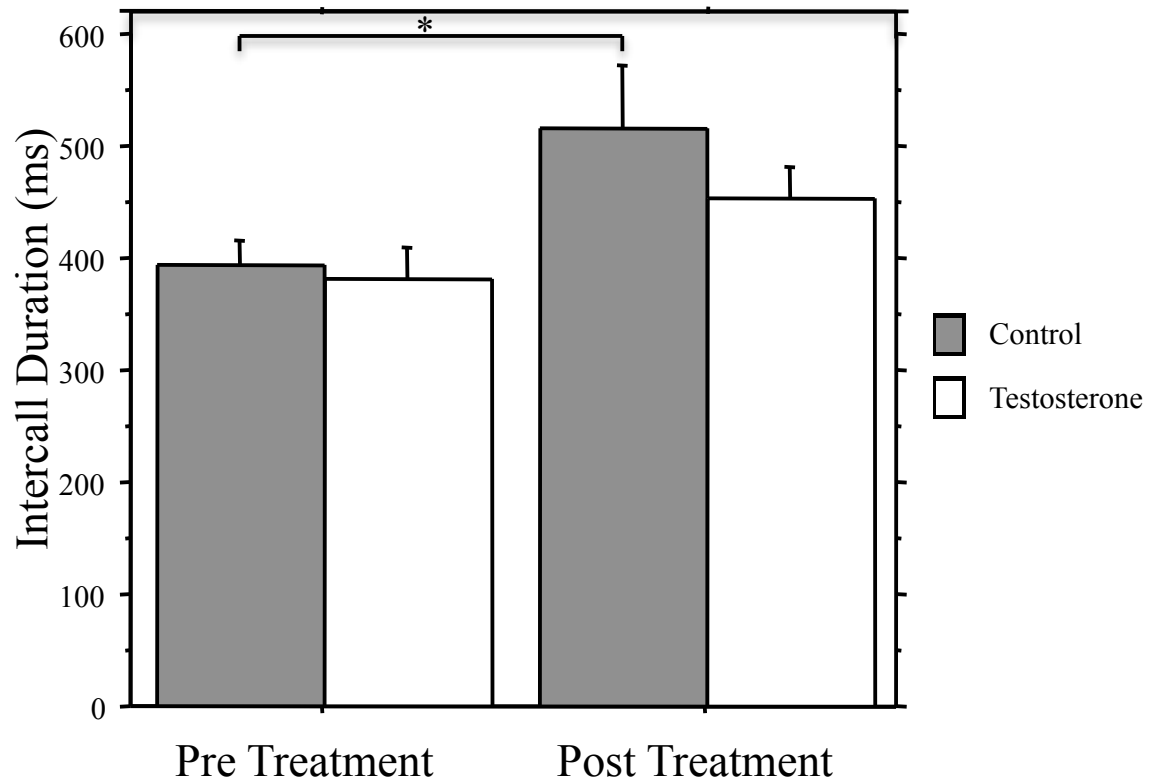


Figure 13: Changes in intercall duration (ms) prior to and subsequent to castor oil (n = 9) or T injection (n = 10) in *H. cinerea*. Whiskers depict standard errors and asterisks denote significant differences.

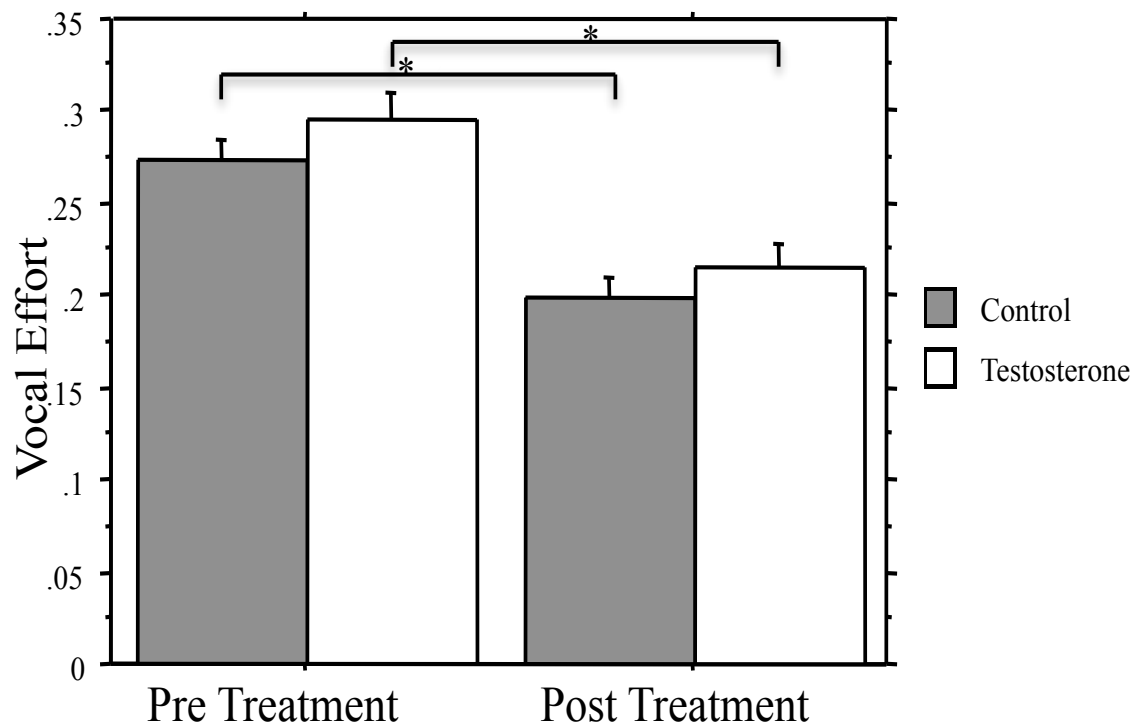


Figure 14: Changes in vocal effort prior to and subsequent to castor oil (n = 9) or T injection (n = 10) in *H. cinerea*. Whiskers depict standard errors and asterisks denote significant differences.

## DISCUSSION

There was no evidence that vocal effort increased in calling male *H. cinerea* subsequent to T injections, despite the fact that injections resulted in a significant elevation of circulating T levels compared to castor oil-injected males. Thus, my results provide little support that androgens have a graded effect on vocalization, contrary to predictions of the ICHH (Folstad and Karter, 1992) or the EHV model (Emerson, 2001). The data suggest that androgens act on vocalization via threshold effects where elevations in circulating androgen levels are necessary for vocal behavior (*reviewed* in Wilczynski et al., 2005), but any increase in androgen levels above threshold does not affect the extent of vocalization.

Hormone concentrations were analyzed to observe any changes in hormone levels prior to and subsequent to treatment. A significant decrease in circulating DHT levels was observed in males injected with T and castor oil. This decrease may be explained by concepts of the EHV model, which predicts that conspecific male vocalizations stimulate androgen production in male signal receivers (Emerson, 2001). By removing males from their acoustic environment (as was done during treatment), a decrease in DHT may have occurred because of the lack of auditory stimulation from chorusing males. Even though levels of CORT did not increase significantly in T-injected and castor oil males, a slight increase in CORT (i.e., in T-injected individuals, see Fig. 9C) may have altered vocal behavior. For example, in Great Plains toads, *Bufo cognatus*, elevations in CORT levels mediate a decrease in call duration (i.e. vocal effort) and cause males to cease

vocalization entirely, independent of changes in circulating androgens (Leary et al., 2004, 2006, 2009). Hence, a small increase in CORT levels may have mediated a decrease in vocal effort, despite elevating levels of T. Further studies need to be done to assess the effects of CORT on vocalization in *H. cinerea*.

Results from vocal analysis did not provide any evidence that vocal effort increased with increasing levels of T. High and low carrier frequencies are under stabilizing selection via mate choice by females and are not predicted to vary with circulating levels of T. The data indicated that high carrier frequency did not differ significantly between T and castor oil-injected males. However, there was a significant increase in low carrier frequency subsequent to T injections. This increase in low carrier frequency may be a consequence of the effects of T on musculature associated with vocalization (i.e., trunk muscles and larynx). Emerson (2001) suggested that increasing levels of T causes the musculature to contract with greater force. Such an increase in contractile force causes air to be ejected over the vocal cords with greater intensity and could thus increase low carrier frequency. However, linear regression results indicated that there was no correlation between T levels and low carrier frequency prior to treatment (Fig. 4). Further investigations are needed to verify that the increase in low carrier frequency is a result of increasing levels of T.

Gerhardt (1991) demonstrated that call duration and intercall duration are under directional selection via mate choice by females and thus are predicted to vary with levels of androgens. Female *H. cinerea* are known to prefer calls that are longer and faster than the mean of the population (Gerhardt, 1991). Surprisingly, call duration decreased significantly in males injected with T and intercall duration increased (i.e., call rate

decreased) in control males; thus, both groups decreased their overall vocal effort (Fig. 14).

The ICHH and EHV model predict that female selection for elaborate traits drives concordant directional selection on circulating androgen levels. My results provided no evidence of a graded effect of testosterone on vocalization. Hence, elevated levels of circulating T does not appear to mediate changes in vocalization, contrary to the predictions of the ICHH and EHV model. My research suggests that T mediates vocal behavior via a threshold (non-linear) effect in green treefrogs. In some organisms, however, androgens alter the expression of elaborate male traits in a graded manner. For example, in red jungle fowl, androgen administration results in an increase in a male's comb length (Zuk et al., 1995). It is currently not clear why androgens affect the expression of elaborate male traits in a graded manner in some species but not in others. Additional work is clearly needed to address this problem.

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